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(54) **CAPILLARY ELECTROPHORETIC METHODS TO DETECT NEW BIOLOGICALLY ACTIVE COMPOUNDS IN COMPLEX BIOLOGICAL MATERIAL**

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(58) **Field of Search** 204/601, 602, 204/603, 604, 605, 451, 452, 453, 455, 606, 607, 608, 610, 612, 615, 616, 617, 618, 621; 436/516, 536, 538, 540

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(57) **ABSTRACT**

This invention relates to a competitive-binding, capillary electrophoretic method of detecting new therapeutic regulatory (modulating) and diagnostic compounds in natural samples and other complex biological materials. The present method generally comprises mixing a preselected, detectable target with a sample of complex biological material to produce a first, sample/target mixture capillary electrophoresis apparatus. Subsequently, the first mixture is mixed with a pre-selected, light-binding competitive ligand (TBCL), prior to produce a second, sample/target/TBCL mixture, for a predetermined optional incubation period sufficient to allow the TBCL to bind a pre-selected percentage of the available target in the absence of any other ligand. An aliquot of the second mixture is subsequently subjected to pre-optimized capillary electrophoresis, during which the migration of the target is monitored. The presence of a potential new compound is indicated by the increase in the peak area of the unbound target peak and/or decrease in the peak area of the TBCL/target complex peak. A capillary electrophoretic profile of the second mixture is produced, which may be compared to a reference standard. The reference standard typically comprises a capillary electrophoretic profile or migration pattern of the target when mixed with a TBCL in an absence of any other competing ligand under similar, pre-selected capillary electrophoretic conditions.

32 Claims, 11 Drawing Sheets

CAPILLARY ELECTROPHORETIC METHODS TO DETECT NEW BIOLOGICALLY ACTIVE COMPOUNDS IN COMPLEX BIOLOGICAL MATERIAL

CROSS-REFERENCE TO RELATED APPLICATION(S)

This application claims the benefit of Provisional Application No. 60/069,943, filed on Dec. 17, 1997, herein incorporated by reference.

FIELD OF THE INVENTION

This invention relates to screening complex biological material for new biologically active compounds, and in particular, to using capillary electrophoresis for such screening.

BACKGROUND OF THE INVENTION

Developing screens to identify new biologically active compounds can present unique and difficult challenges, especially when screening naturally occurring complex biological materials (sometimes referred to as "natural samples" or "natural products"), various biological preparations, chemical mixtures, and other complex materials. Major problems include low concentrations of active compounds, unknown components that can interfere with screening agents, and isolation of the new compound once a positive sample is obtained. Despite these obstacles, the pharmaceutical industry still maintains a strong interest in the screening of complex mixtures. For example, it is widely recognized that nature provides a virtually endless supply of new chemical structures that are often difficult or impossible to synthesize in a cost-effective manner. Most natural products have some bio-activity, and historically, natural products and their analogs have been the most successful source of therapeutic compounds.

Screening technologies for therapeutic and other biologically active compounds fall into two broad categories: bioassays and mechanism-based assays (Gordon et al., *J. Med. Chem.* 37:1386–1401, 1994).

Bioassays represent the oldest, and so far, most productive screening tool. Bioassays measure the effect of natural samples on the viability or metabolism of disease-related cell types such as bacteria, fungi, viruses, and tumor cells. For example, the β-lactam antibiotics (e.g., penicillins and cephalosporins) were discovered by testing microbial broths for bacterial growth inhibition in culture tests. Likewise, the antifungal compounds, nystatin and amphotericin B, were isolated from broths that inhibited yeast growth in culture tests. However, mainly due to the lack of specificity and sensitivity of bioassays, the more sophisticated mechanism-based assays have replaced most bioassays as primary screens.

Mechanism-based assays can be subdivided into three general categories: (1) recombinant cell-based assays, (2) enzymatic/biochemical assays, and (3) binding assays. Today's assays must satisfy the need for high throughput capacity, so they must be robust, simple, and amenable to automation in a parallel processing mode.

Recombinant, cell-based assays screen for a given, known functional response. Usually a target receptor, enzyme, or other protein is introduced into cultured cells by genetic engineering. Inhibition or induction of target activity is associated with an easily-measured response. For example, modifiers of transcription factors (TF) can be measured by

fusing the TF's target DNA sequence (typically an enhancer or promoter region) to a luciferase (light-producing) gene. TF agonists result in transcription of the luciferase gene, and light is produced. If an antagonist is present, light is not produced. One advantage of cell-based assays over enzymatic and binding assays is that they may provide more physiologically appropriate leads, because intact cells are used. On the other hand, cell-based screens can be very difficult to develop, slow and quite variable in their results (Janzen et al., *Society for Biomolecular Screening Meeting*, Nov. 7–10, 1995).

Enzymatic assays are cell-free screens that directly or indirectly test the effect of soluble compounds on the activity of purified target enzymes that are related to disease processes. For example, viral reverse transcriptase inhibitors can be screened by measuring the incorporation of radiolabeled thymidine into a growing DNA chain from a polyuridine RNA template. These assays can be very sensitive and are amenable to automation using microtiter plates. For natural product screening, however, unknown compounds in the samples can dramatically interfere with screening results, leading to unacceptably high levels of false negatives and false positives. For example, greater than 15% of aqueous extracts from terrestrial plants, cyanobacteria, marine invertebrates, and algae exhibit positive activity in screens for anti-HIV compounds, due to interfering compounds such as plant tannins (Cardellina et al., *J. Nat. Prod.* 56:1123–1129, 1993).

Binding assays are particularly useful for screening soluble mixtures of biological or chemical materials for compounds that bind, and thus potentially modulate or inhibit, physiologically active target molecules. These assays have been major screening tools in the drug discovery efforts of pharmaceutical and biotechnology companies. In immobilized-target binding assays, the target molecule (usually a protein) can be affixed or tethered to a solid substrate such as the side of a microtiter well, beads, or chromatographic supports. If the target molecule is a receptor, it can be expressed on the membrane of a cell attached to the solid support. The samples are incubated with the immobilized targets, and bound ligands are detected, usually through an associated calorimetric or fluorescent reaction. Alternatively, the sample is mixed with a soluble-phase target that is captured using an anti-target antibody. Such binding assays are advantageous because they facilitate the washing and isolation of target-ligand complexes.

However, immobilized-target binding assays also suffer from several disadvantages, particularly as a method for screening natural biological samples for new active compounds. One problem is that the binding of multiple background compounds, if present in sufficient quantities, may produce a positive signal that is indistinguishable from that of a single potential therapeutic compound. Therefore, screening with immobilized-target binding assays often requires heavy washing or improved clean-up capability. Another general problem is that affixing target proteins to solid substrates often inactivates the protein or produces a functional change. This problem can be addressed to some extent by using recombinant DNA technology to insert an inert "handle" such as a peptide epitope into the target protein. The protein-ligand complex can then be isolated through the use of an antibody to this epitope. However, development of these modified targets is time-consuming and expensive.

One commonly used binding assay is the microtiter-format, enzyme-linked immunosorbent assay (ELISA). One disadvantage is that the target molecule, which is usually

at 6.670 minutes, which was much larger than the reduced LTG/DZ peak. The unbound LTG peak indicated the presence of an unknown hit compound in the screened natural extract sample that could bind to LTG. Also observed was a series of peaks spread broadly over the region of 2.5–3.5 minutes, representing unknown fluorescent peaks in the natural extract. This result points out the sensitivity and power of the present method. If this screen had not been a CE separation technique and had been done in a microtiter well, the fluorescent background peaks would have been undistinguishable from a target peak and would have given a false positive (or negative) result in a fluorescence-based binding assay.

EXAMPLE III

FIG. 6 depicts the CE results of another test run of the present screening assay, under similar conditions as in Example I, except that the screened inert sample contained 10 nM (instead of 100 nM) of the test hit compound EZ. As well, two different pre-CE incubation times with the TBCL were tested.

Panel [A] of FIG. 6 depicts the CE profile of 5 nM LTG after a 1-minute incubation with 2 nM DZ. As before, a major peak corresponding to the TBCL/DZ complex is seen at 11.987 minutes. A minor peak corresponding to unbound, LTG is seen at 11.727 minutes.

In panel [B] of FIG. 6, 5 nM of LTG was incubated first with an inert natural sample spiked with 10 nM of ethoxyzolamide (EZ) for 10 minutes, and then with 2 nM dorzolamide (DZ) for 1 minute. Due to competitive binding of the target by the test hit compound, EZ, the LTG/DZ complex peak at 12.033 minutes was reduced, while the unbound target peak at 11.763 minutes was higher. Therefore, prior binding of the EZ to the LTG prevented some, but not all, LTG from binding to the DZ during the later, 1-minute incubation with DZ.

Panel [C] of FIG. 6 shows the capillary electrophoresis profile of a sample of the same mixture as in Panel [B] of FIG. 6, (5 nM LTG with 10 nM EZ-containing natural sample, and then with TBCL, 2 nM DZ). However, the mixture is subjected to an additional 20 minutes of incubation time with DZ, prior to CE. That extra incubation time allowed the tight-binding DZ ligand to almost completely displace bound EZ from the LTG prior to injection into the CE apparatus. Therefore, the resulting. CE profile showed a higher target/LTG/DZ complex peak at 12.020 minutes and a greatly reduced peak of unbound LTG at 11.753 minutes (in comparison to panel [B] of FIG. 6). Thus, as demonstrated, this method can be used to show that a detectable unknown hit compound specifically competes with the TBCL. For example, one could claim that a screened natural sample displaying a "hit"—e.g., a sample that alters target mobility by producing a difference in peak area, location, or shape—is simply destroying the activity of some of the target (LTG), which thus can no longer bind the TBCL (DZ). Panel [C] of FIG. 6 proves that the LTG remains functionally active during the screening method, because given enough incubation time (of the sample/LTG/DZ mixture), DZ replaces the test hit compound EZ in binding to LTG.

EXAMPLE IV

FIG. 7, panel [A] depicts the CE profile of 5 nM LTG incubated with 2 nM DZ, under the same conditions as for FIG. 4, panel [B]. A major peak corresponding to the LTG/DZ complex was seen at 7.080 minutes. A minor peak corresponding to unbound LTG was seen at 6.947 minutes.

Panel [B] of FIG. 7 shows the CE profile of 5 nM LTG after incubation with a natural sample that contains an unknown hit compound and then with the TBCL, DZ, under the same conditions as above. The LTG/DZ complex peak at 7.260 minutes was reduced, while the unbound LTG peak at 7.110 minutes was higher.

Panel [C] of FIG. 7 shows the CE profile of an aliquot of the same mixture as for FIG. 7, panel [B], after an additional 40 minutes of incubation with DZ prior to injection into the CE apparatus. DZ has displaced some but not all of the hit compound from being bound to LTG, and a higher LTG/DZ complex peak is seen at 7.277 minutes and a reduced unbound LTG peak is seen at 7.127 minutes. These results show that the screening assay has detected a tight-binding hit, or a moderate-to-weak binding hit at a high concentration, since DZ was unable to completely replace the hit compound even after another 40 minutes of incubation (as opposed to FIG. 7, panel [A]'s 1-minute incubation).

EXAMPLE V

This example illustrates the case where a tight-binding hit compound that has a different charge from, or a higher MW than, the TBCL, is present in the natural sample.

Panel [A] of FIG. 8 depicts the CE profile of 5 nM LTG incubated with 2 nM DZ. A major peak corresponding to the LTG/DZ complex is seen at 7.143 minutes. A minor peak corresponding to the unbound LTG is seen at 6.997 minutes.

Panel [B] of FIG. 8 shows 5 nM LTG incubated with a natural sample that contains an unknown, tight-binding hit compound, and then incubated with 2 nM DZ for the optimal incubation time. As seen in the figure, most of the LTG was complexed to DZ because the LTG/DZ peak at 7.173 minutes is still large. The minor unbound LTG peak at 7.017 minutes is still present. However, a new peak is now visible on the left-hand shoulder of the unbound LTG peak. This shoulder peak is a stable complex of LTG with an unknown hit compound in the natural sample that has a different (opposite) charge from that of the TBCL (DZ). The opposite charge on the unknown hit makes the complex appear at an earlier time than the unbound LTG, rather than at a later time like the LTG/DZ complex.

This screening method has successfully detected a wide variety of hit compounds having different charges (negative, neutral, or positive) and different binding affinities to the target molecule, human carbonic anhydrase II (HCA-II). For instance, it has detected moderately binding hit compounds such as 0.5 nM of ethoxyzolamide and 2.0 nM of dichlorphenamide. Weak-binding hit compounds detected include 80 nM of acetazolamide and 150 nM of methazolamide. The method has also detected a very weak-binding hit compound, carzenide (PCBS) at a 30,000 nM concentration. See, e.g., FIG. 9 for calibration curves of different concentrations of different hit compounds binding to the HCA-II target.

While the present invention has been described in conjunction with preferred embodiments, one of ordinary skill in the art, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein.

What is claimed is:

1. A method of screening complex biological material for previously unidentified, weak-to-tight-binding hit compounds that bind to a pre-selected, detectable target, comprising, in the order given, the steps of:

(1) providing a sample of complex biological material;